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Short Communication

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First Report of *Calonectria ilicicola* Causing a New Disease on Laurus (*Laurus nobilis*) in Europe

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Abstract

Crown and root rot has been detected on potted *Laurus nobilis* plants in a nursery located in the Catania province (Italy). Perithecia referable to a *Calonectria* species were consistently detected on crowns and stems of symptomatic plants. Based on morphology, cultural features and molecular analysis, the species was identified as *Calonectria ilicicola*. Koch's postulates were fulfilled by pathogenicity tests carried out on potted *Laurus nobilis* seedlings. To our knowledge, this is the first report of the occurrence of a disease caused by *Ca. ilicicola* on *Laurus nobilis*.

Introduction

During 2009 and 2010, a new disease was observed on approximately 10% of 10 000 2-year-old potted bay laurel (*Laurus nobilis* L., Lauraceae family) plants in a nursery located in Mascali (Giarre, Catania province, eastern Sicily, Italy). Infected plants showed internal large brown areas with necrotic tissues at the crown level, the basal stem and root rots. Death of bay laurel potted plants as a consequence of the symptoms above mentioned was observed. Orange to red perithecia referable to *Calonectria* sp. were frequently detected in groups on the crowns and basal stems of symptomatic plants (Fig. 1). Brayford and Chapman (1987) reported a wilting disease of *L. nobilis* in nurseries on the Isles of Scilly (UK). The causal agent was identified as *Cylindrocladium ilicicola* (Hawley) Boedijn & Reitsma, but incorrectly linked to the teleomorph name *Ca. ilicicola* Boedijn & Reitsma. Based on a molecular comparison of ex-type strain, Crous et al. (1993) showed *Ca. ilicicola* is the teleomorph of *C. parasiticum* Crous, M.J. Wingf. & Alfenas. More recently, Lechat et al. (2010) proposed *Ca. laurii* (Vanderw.) Lechat & Crous as the teleomorph name of *C. ilicicola*. The aim of the present study was to identify the *Calonectria* species involved on bay laurel in Italy with both morphological and molecular characterization and to verify the pathogenicity of this fungus.

Materials and Methods

Isolations were performed transferring fragments of symptomatic crown, stem and root tissues of *L. nobilis*, surface-sterilized with 1% NaClO for 1 min, on potato dextrose agar (PDA). Petri dishes were incubated at 25°C in the dark. For the morphological identification of the anamorph stage, single hyphal tip or single viable ascospore were transferred on malt extract agar (MEA), carnation leaf agar (CLA) and synthetic low-nutrient agar (SNA). Because we were unable to induce the formation of the anamorph stage on different media, ascospore suspension (1.0×10^4 - ascopores/ml) obtained from 30-day-old cultures grown on CLA was used for the inoculation of red clover (*Trifolium pratense* L.) seedlings. Morphological characteristics of the anamorph and teleomorph stages of twenty isolates were examined. Total genomic DNA was extracted from five representative 7-day-old single-conidial isolates using the UltraClean™ Microbial DNA isolation kits (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) as described by the manufacturer. Partial gene sequences were determined for calmodulin (CAL), histone H3 (HIS3) and translation elongation factor 1-α (TEF-1α), as described by Lombard et al. (2010), and the amplicons were sequenced in both directions using the same PCR primers. Sequence data

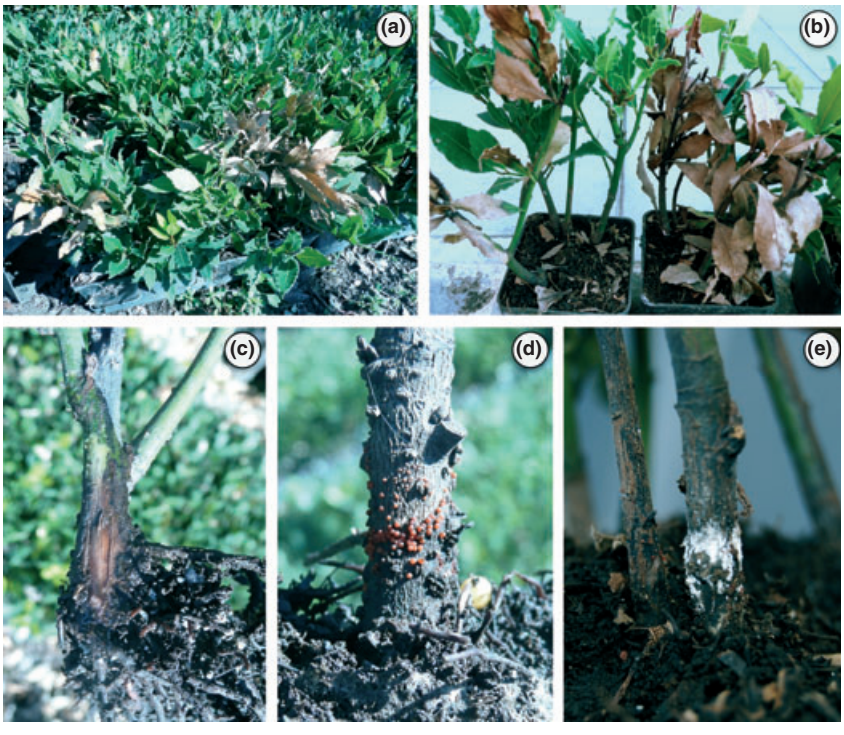

Fig. 1 Death of *Laurus nobilis* potted plants as a consequence of crown rot and root rot caused by *Calonectria ilicicola* (a–b); Brown discolouration of the basal stem (c); Perithecia of *Ca. ilicicola* (d); Mycelia of *Cylindrocladium parasiticum* (e)

Table 1
Sequences of *Calonectria* spp. used in the study

Species	Accession number[a]	GenBank number		
		Calmodulin	Histone H3	TEF-1α
Calonectria asiatica	CBS 112711; CPC 3898; SFE 744	AY725738	AY725655	AY725702
	CBS 114073[b]; CMW 23782; CPC 3900; SFE 726	AY725741	AY725658	AY725705
Calonectria chinensis	CBS 112744; CMW 30986; CPC 4104	AY725746	AY725660	AY725709
	CBS 114827[b]; CMW 23674; CPC 4101	AY725747	AY725661	AY725710
Calonectria colombiensis	CBS 112220[b]; CMW 23676; CPC 723	AY725748	AY725662	AY725711
	CBS 112221; CMW 30985; CPC 724	AY725749	AY725663	AY725712
Calonectria curvispora	CBS 116159[b]; CMW 2369; CPC 765	GQ267374	AY725664	GQ267302
Calonectria hongkongensis	CBS 114711; CMW 30995	AY725754	AY725666	AY725716
	CBS 114828[b]	AY725755	AY725667	AY725717
Calonectria ilicicola	CBS 190.50[b]; IMI 299389; CMW 30998; CPC 2482	AY725764	AY725676	AY725726
	CBS 115897	GQ267403	GQ267256	GQ267321
	DISTEF-LN1; CBS 129185	JF714949	JF714954	JF714959
	DISTEF-LN3; CBS 129186	JF714950	JF714955	JF714960
	DISTEF-LN5; CBS 129187	JF714951	JF714956	JF714961
	DISTEF-LN6; CBS 129188	JF714952	JF714957	JF714962
	DISTEF-LN11; CBS 129189	JF714948	JF714953	JF714958
Calonectria indonesiae	CBS 112823[b]; CMW 23683; CPC 4508	AY725756	AY725668	AY725718
	CBS 112840; CPC 4547	AY725758	AY725670	AY725720
Calonectria kyotensis	CBS 170.77; IMI 299388; CMW 23679	GQ267380	GQ267249	GQ267308
	CBS 413.67; CMW 23678; CPC 2391	GQ267379	GQ267248	GQ267307
Calonectria malesiana	CBS 112710; CPC 3899	AY725759	AY725671	AY725721
	CBS 112572[b]; CMW 23687; CPC 4223	AY725760	AY725672	AY725722
Calonectria multiphialidica[c]	CBS 112678; CMW 23688	AY725761	AY725673	AY725723
Calonectria naviculata[c]	CBS 101121[b]; CMW 30974	GQ267399	GQ267252	GQ267317
Calonectria pacifica	CBS 109063[b]; IMI 35428; CMW 16726; CPC 2541	AY725762	GQ267255	AY725724
	CBS 114038; CMW 30988	GQ267402	AY725675	GQ267320
Calonectria sumatrensis	CBS 112829[b]; CMW 23698; CPC 4518	AY725771	AY725696	AY725733
	CBS 112934; CMW 30987; CPC 4516	AY725773	AY725698	AY725735

[a]CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; CMW: Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CPC: Pedro Crous Working collection housed at CBS.
[b]Ex-type culture.
[c]*Calonectria* spp. used as outgroup in the phylogenetic analysis.
Bold values indicate Ca. ilicicola isolates, principal subject of this paper.

from Lombard et al. (2010) were used as reference data, and subsequent alignments were generated as described in Lombard et al. (2010) (Table 1). Nucleotide substitution models were determined for each locus using the Akaike information criterion in MrModeltest v. 2.2 (Nylander 2004). A phylogenetic tree with Bayesian probabilities for the combined data set was generated using the Markov Chain Monte Carlo (MCMC) algorithm of MrBayes v. 3.1.1 (Ronquist and Heulsenbeck 2003). The four chains of the MCMC analysis were run simultaneously for one million generation from random trees. The posterior probabilities were determined after the first 1000 trees were discarded as the burn-in phase. A maximum-parsimony genealogy was also done using PAUP v. 4.0b10 (Swofford 2002). The heuristic search was based on 1000 random addition sequences and tree bisection–reconnection, with the branch swapping option set on 'best trees' only. Alignment gaps were treated as missing data, and all characters were weighted equally. Branch support was determined using bootstrap analysis based on a 1000 replications. Pathogenicity of the five representative isolates used in the molecular analysis was confirmed by applying 5 ml of a ascospore suspension (1.0×10^4 ascospores/ml), obtained on CLA, to the crowns of potted 1-month-old seedlings of bay laurel that were subsequently covered with plastic bags and maintained in a growth chamber (23–25°C). Twenty plants for each isolate were used. The same number of control plants were treated with water.

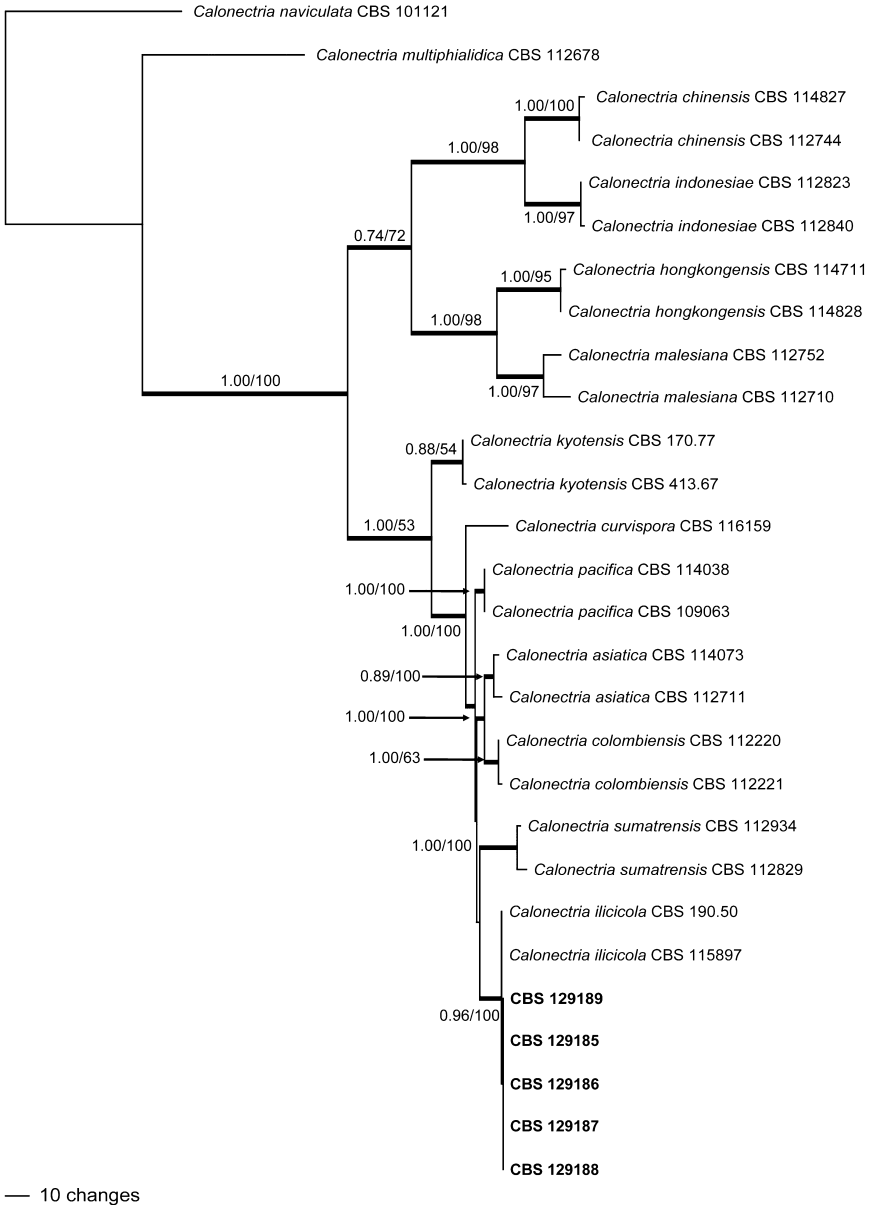

Fig. 2 The most parsimonious tree obtained from a heuristic search with a 1000 random additions sequences for the combined calmodulin (CAL), HIS3 and TEF sequences alignments. Scale bar shows 10 changes, and posterior probabilities/bootstrap supports are indicated at the nodes. Thickened lines indicate branches also present in Bayesian consensus tree

Results and Discussion

A *Calonectria* sp. was consistently obtained from symptomatic tissues of bay laurel. On PDA, colonies were fast growing with abundant white, floccose, aerial mycelia and reverse salmon-buff colour. Conidia were cylindrical, rounded at both ends, straight, ranged from 40 to 50 μm long × 4 to 5 μm wide (mean = 44.8 × 4.6 μm), 1(−3) septate and were produced only on red clover seedlings. Stipe extension was terminating in sphaeropedunculate vesicles. Perithecia were produced after 20–30 days in all media and occurred solitary or in groups, orange to red–brown, subglobose to ovoid and ranged from 330 to 460 μm long × 330 to 400 μm in diameter (mean = 402 × 355 μm),. Ascospores were hyaline, fusoid with rounded ends, straight to slightly curved, 1(−3) septate, not or slightly constricted at the septum and ranged from 31 to 45 μm long × 4 to 7 μm wide (mean = 37 × 5.2 μm). Amplicons of approximately 450 bases for HIS3 and 500 bases each for CAL and TEF-1α were determined. The adjusted alignment for each gene region consisted of 28 taxa including *Ca. naviculata* (CBS 101121) and *Ca. multiphialidica* (CBS 112678) as outgroup taxa. The outgroup taxa were selected based on their phylogenetic position as determined by Lombard et al. (2010). A HKY + I + G model for CAL and TEF-1α and a GTR + I + G model for HIS3 was selected for Bayesian analysis. The Bayesian consensus tree obtained confirmed the tree topology obtained with maximum-parsimony including bootstrap support. For the maximum-parsimony analysis, the combined sequence data set of 1431 bases, including alignment gaps, consisted of 1035 constant characters, 121 uninformative characters and 275 parsimony-informative characters. Parsimony analysis yielded one most parsimonious tree (Fig. 2). In the tree, all the isolates isolated from *L. nobilis* grouped with *Ca. ilicicola* in the Sphaero-naviculate group (Lombard et al. 2010) with good bootstrap and posterior probability support. Symptoms identical to those observed in the nurseries developed 10–14 days after the inoculation, and most part of the inoculated plants were killed within 2 months. No variation on virulence degree was observed among the isolates tested. This pathogen was reisolated from the infected tissues and identified as previously described.

Morphological features of the fungus associated with disease symptoms on bay laurel including conidia and perithecial morphology, as well as partial gene sequences for CAL, HIS3 and TEF-1α, identify the isolates studied as *Ca. ilicicola*. In addition, Koch's postulates demonstrated the pathogenicity of this fungus and its role as causal agent of the new disease detected in Italy. *Ca. ilicicola* induces peg, pod and root necrosis of peanuts, but also leaf spot, damping-off, crown root and blight of several hosts (Crous 2002). This species has a large distribution on different countries (Crous 2002). In Europe, *Ca. ilicicola* was previously reported only on *Nerium oleander* in United Kingdom (Crous 2002). To our knowledge, this is the first report of the occurrence of a disease caused by *Ca. ilicicola* on *Laurus nobilis*, and it is the first detection with molecular data of this fungal pathogen in Europe.

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